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Separation and analysis of the glycoform populations of ribonuclease B using capillary electrophoresis

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The development of methods to separate, analyse and monitor changes in glycoform populations is essential if a more detailed understanding of the structure, function and processing of glycoproteins is to emerge. In this study, intact ribonuclease B was resolved by borate capillary electrophoresis into five populations according to the particular oligomannose structure associated with each glycoform. The relative proportions of these populations are correlated with the percentages obtained indirectly by analysis of the hydrazine released oligosaccharides using Bio-Gel P-4 gel filtration, matrix assisted laser desorption mass spectrometry and high performance anion exchange chromatography. Alterations in the composition of the glycoform populations during digestion of ribonuclease B with *A. saitoi* α(1-2)mannosidase were monitored by capillary electrophoresis (CE). Digestion of the free oligosaccharides under the same conditions, monitored by anion exchange chromatography, revealed a difference in rate, allowing some insight into the role of the protein during oligosaccharide processing. In conjunction with other methods, this novel application of CE may prove a useful addition to the techniques available for the study of glycoform populations.

Keywords: Capillary electrophoresis, glycoforms, oligomannose, ribonuclease B.

Glycoproteins generally consist of populations of glycosylated variants of a single protein (glycoforms). Molecules comprising a unique amino acid sequence may be diversified by a range of oligosaccharides covalently bound to the glycosylation sequon they contain. Each potential glycosylation site is normally able to accommodate many alternative oligosaccharide structures, allowing the generation of discrete populations of a structurally modified protein.

The relative proportions of such glycoforms are found to be reproducible, not random, and depend on the environment in which the protein is glycosylated. Factors which control this include the physiological state as well as the type of organism, tissue and cell in which the glycoprotein is made. Manufacturing processes also influence glycosylation and these, as well as procedures for isolating glycoproteins, may result in the inadvertent selection of particular glycoform populations. Such considerations are important not only because glycosylation modifies structure, but because it may also modulate the function of a protein.

Work on tissue plasminogen activator (t-PA) [1] and uromodulin [2] has clearly demonstrated that the different glycoforms of these proteins have differential activities. In general, therefore, a glycoprotein may display a spectrum of activities by virtue of its glycoform populations. Chemical methods have been used to separate glycoproteins into pools of glycoforms with a common functional property. In this work, the novel application of a physical technique, capillary electrophoresis (CE), has enabled the individual glycoforms of ribonuclease B (RNAse B) to be fully resolved according to the number of residues in the oligosaccharide chains associated with each glycoform.

Conventional polyacrylamide gel electrophoresis (PAGE) of glycoproteins frequently results in a diffuse band of poorly separated components. The heat generated in the gels by the high voltages required to separate glycoforms is not easily dissipated and causes band broadening and loss of resolution. Capillary electrophoresis [3] uses silica based capillary tubes instead of gels. The high surface area/volume ratio of the capillary enables the heat to be dissipated efficiently, allowing resolutions between 10^5 and 10^7 theoretical plates to be achieved [4, 5].

The capillary, which is filled with buffer, bridges two buffer reservoirs. When a high voltage (up to 30 kV) is applied, one reservoir becomes the anode and the other the cathode. Samples (approx. 5 ng) are injected into the capillary inlet at the anode and the components are detected

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on line by UV absorbance as they move towards the cathode. Detection at the anode can be arranged by reversing the polarity of the system.

The two factors which govern the movement of ions in the capillary are electrophoretic mobility, which depends on the mass/charge ratio, and electroosmosis, which depends on the pH of the buffer system. The surface of the inner walls of the silica capillaries has a negative charge. When a high voltage is applied and the buffer has a high pH, the layer of electrolyte in contact with the wall becomes positively charged and moves to the cathode together with the medium of the buffer. In contrast to electrophoretic mobility, electroosmotic flow carries all substances injected at the anode towards the cathode. Positively charged species will therefore be accelerated towards the cathode. At neutral or high pH, electroosmotic flow generally exerts the stronger influence; at low pH the electrophoretic flow prevails.

Matrix assisted laser desorption mass spectrometry [6] is a relatively new technique which provides a rapid and sensitive means of analysing underivatized oligosaccharides. Previously, derivatization has been an essential stage in the majority of mass spectrometric protocols for oligosaccharide analysis, either to produce a volatile derivative for ionization by electron impact, or to improve sensitivity. This technique overcomes the need for derivatization and uses matrices such as 2,5-dihydroxybenzoic acid or 3-amino-4-hydroxybenzoic acid [7]. A typical sample loading is 1 pmol and the analysis time around 5 min, depending on the number of summed laser shots required to give an acceptable signal-to-noise ratio.

In this work, separation of the glycoforms of RNAse B has been achieved through the formation of anionic borate complexes with *cis* hydroxyl groups. Since the glycoforms differ in the number of groups they contain which have the correct conformation to form borate complexes, such complexes may be separated from each other on the basis of charge. Experiments on free sugars have demonstrated that there is no direct correlation between the number of *cis* diol groups and the elution time of the borate complexes on CE [8] and that, therefore, other constraints can also apply. Nevertheless, borate buffer has been used to resolve free derivatized [8, 9] sugars by CE, and has been used to resolve isoforms, for example, of human transferrin [10]. In this case, it has been applied to the separation of glycoforms.

Materials and methods

Release and radioactive labelling of asparagine linked oligosaccharides from RNAse B

100 nmol RNAse B (bovine pancreas X11B; Sigma Chemical Co., St. Louis, MO, USA) was cryogenically dried over activated charcoal and subjected to hydrazinolysis, re-*N*-acetylation, and reduction with NaB^3H_4 according to the standard procedures in this laboratory [11]. The reduced and radiolabelled sugars were purified by paper chromatography and high voltage electrophoresis, and finally passed through a tandem column of Chelex 100 (Na^+)/Dowex AG50-X12 (H^+)/AG3-X4A (OH^+)/QAE Sephadex A-25 in water to remove metal ions and salt.

P-4 gel filtration

A volume of eluate equivalent to 2 nmol of radiolabelled sugar (2×10^6 counts min^{-1}) was evaporated to dryness and redissolved in 180 μl water. Dextran hydrolysate (20 μl; 20 mg ml^{-1}) was added, and the mixture applied to a Bio-Gel P-4 (>400 mesh; Bio-Rad Labs, Richmond, CA, USA) gel permeation chromatography column (1.5 cm × 200 cm).

The eluant was monitored for radioactivity by using an LB503 Berthold HPLC radioactivity monitor, and for refractive index by using an Erma ERC 7510 refractometer. The P-4 chromatograms show radioactivity (vertical axis) plotted against retention time. The numerical superscripts refer to the elution position of the glucose oligomers in glucose units as detected simultaneously by the refractive index monitor (data not shown). Sample elution positions (in glucose units) were calculated by cubic spline interpolation between the internal standard glucose oligomer positions and the relative proportions of the oligosaccharides obtained by integration.

Mass spectrometry

The relative proportions of the oligomannose structures associated with RNAse B were determined by matrix assisted laser desorption mass spectrometry with time-of-flight detection on a Finnigan LASERMAT instrument. 20 pmol RNAse B sugars dissolved in 1 μl water and 500 pmol 3-amino-4-hydroxybenzoic acid dissolved in 0.5 μl water were mixed on the gold spot in the centre of the stainless steel target and air dried.

The data were obtained by the accumulation of data from eight shots from a UV laser (337 nm).

High performance anion exchange chromatography (HPAEC)

HPAEC with radioactive detection can be used to resolve closely related oligosaccharides such as the Man_9–Man_5 series [12]. The chromatography was performed on a Dionex PA1 4 mm × 250 mm column eluted with 150 mM NaOH/30 mM sodium acetate at a flow rate of 1 ml min^{-1}. Detection was by radioactivity monitor (Berthold LB507A). The peaks were assigned using standard markers.

Capillary electrophoresis

A 72 cm fused silica capillary, ID 75 μm, was used; voltage conditions were 1 kV for 1 min, 20 kV for 19 min, 30 °C,

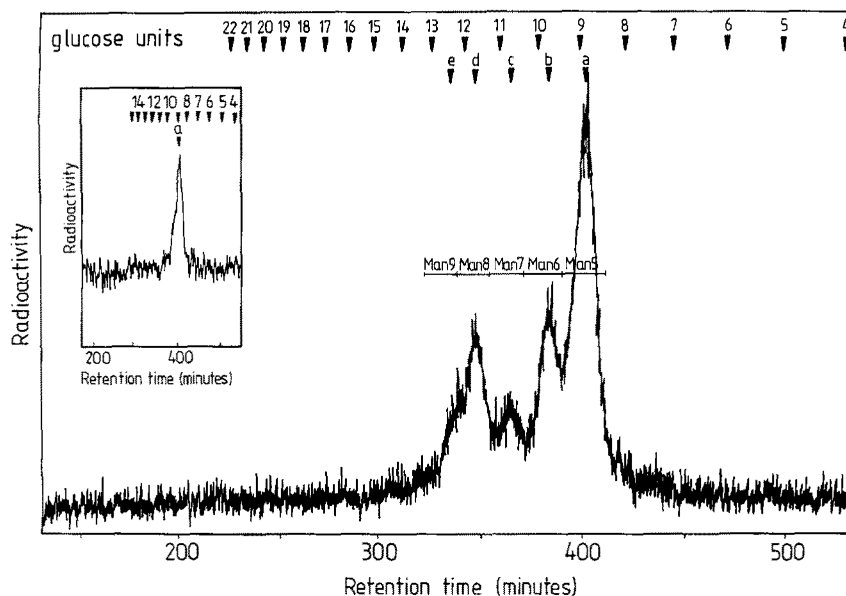

Figure 1. Bio-Gel P-4 (>400 mesh) high resolution gel permeation chromatogram of the N-linked oligomannose oligosaccharides released by hydrazinolysis from ribonuclease B and radiolabelled by reduction with NaB^3H_4. The trace shows radioactivity (vertical axis) plotted against retention time. The numbers refer to the elution volume of glucose oligomers in glucose units, as detected simultaneously by the refractive index monitor (data not shown). Peaks a–e indicate the elution times of the oligomannose series Man_5 (a) to Man_9 (e). Oligosaccharide (a), which results from the digestion of the ribonuclease sugars with *A. saitoi* α(1-2)-mannosidase, elutes at 9 glucose units, consistent with the Man_5 structure which contains only Manα(1-6) and Manα(1-3) residues attached to the *N*-acetylglucosamine core.

wavelength for detection 200 nm, injection time 1.5 s, and the equilibration buffer was 20 mM sodium phosphate, 50 mM sodium dodecylsulphate (SDS), 5 mM sodium tetraborate, pH 7.2. The instrument used was Model 270A from Applied Biosystems Inc.

Exoglycosidase digestion of N-linked oligomannose sugars released from ribonuclease B by hydrazinolysis

33 nmol reduced and radiolabelled sugars were dissolved in 280 μl water and incubated with 250 μl (50 mU ml^{-1}) *A. saitoi* α(1-2)mannosidase in 50 mM sodium acetate buffer, pH 5, containing 1 mg ml^{-1} bovine serum albumin (BSA) at 37 °C. 33 μl aliquots were removed from the reaction mixture at intervals of 0–25 h, boiled immediately to inactivate the enzyme, and stored frozen at −20 °C. Each sample was then desalted on a mixed bed ion exchange resin composed of Chelex, AG50, AG3 and, finally, QAE. The resin columns were washed with water and the eluates evaporated to dryness at 19 °C on a rotary evaporator. The samples were redissolved in 100 μl water and analysed by high performance anion exchange chromatography.

The relative proportions of the released oligosaccharides were determined by integration of these data at time zero.

Exoglycosidase digestion of oligomannose structures present on ribonuclease B and subsequent analysis by CE

56 μg (33 nmol) of ribonuclease B were dissolved in 28 μl of 50 mM sodium acetate pH 5 containing 50 mU ml^{-1} *A. saitoi* α(1-2)mannosidase and 1 mg ml^{-1} BSA. The mixture was incubated at 37 °C and 3 μl aliquots removed at intervals between 0 and 25 h. These were immediately diluted with 9 μl 20 mM sodium phosphate, 50 mM SDS, 5 mM sodium tetraborate, pH 7.2, and applied to a capillary electrophoresis system equilibrated in the same buffer under the conditions described previously.

The relative proportions of the glycoform populations in the intact glycoprotein were obtained by integration of these data at time zero.

Results

The sizes and relative proportions of the oligosaccharide structures released from RNAse B by hydrazinolysis were determined by P-4 gel filtration (Fig. 1), mass spectrometry (Fig. 2) and HPAEC (Fig. 3(a)). The mixture of oligosaccharides was digested with *A. saitoi* α(1-2)mannosidase to a single structure eluting at nine glucose units on P-4 (Fig. 1, insert) and at 4.2 min on HPAEC (Fig. 3(c)).

The intact glycoprotein was resolved by CE into five populations (Fig. 4). The relative proportions of these correlated with the relative proportions of the Man_9–Man_5 oligosaccharide populations determined previously (Table 1). This suggests that CE has resolved RNA'se B into five populations of glycoforms each characterized by one of the oligomannose structures Man_9–Man_5.

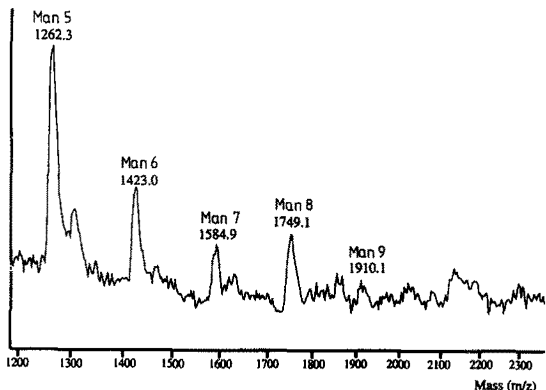

Figure 2. Matrix-assisted laser desorption mass spectrometry data of released sugars from ribonuclease B. The data were obtained using a time-of-flight instrument (Finnigan LASERMAT) equipped with a 337 nm UV laser.

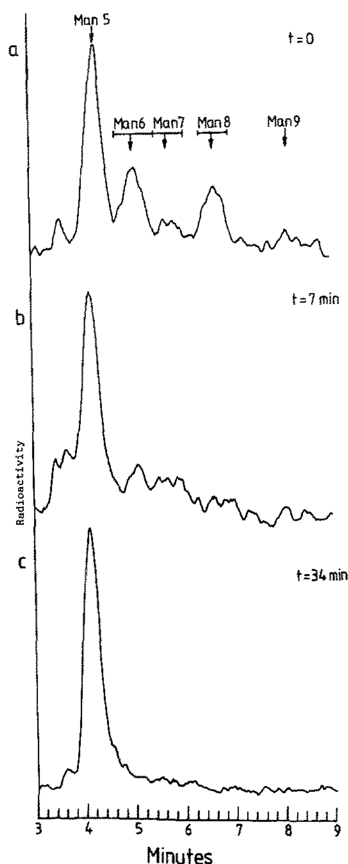

Figure 3. (a) Anion-exchange chromatogram (Dionex-Carbopac PA1) of the N-linked oligomannose oligosaccharides released by hydrazinolysis from ribonuclease B and radiolabelled by reduction with NaB^3H_4. Radioactivity (vertical axis) is plotted against elution time. (b) The same oligosaccharide library after digestion for 7 min with *A. saitoi* α(1-2)mannosidase. (c) After 34 min all oligomannose structures have been reduced to Man_5, detected by the ^3H at the reducing terminal.

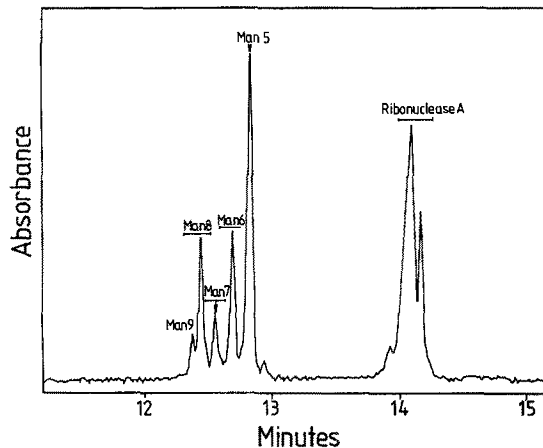

Figure 4. CE profile of ribonuclease showing the nonglycosylated form of the protein, ribonuclease A, and the glycoforms of the same protein, collectively known as ribonuclease B. Ribonuclease A is a contaminant of the ribonuclease B as supplied by Sigma. Nonglycosylated ribonuclease A remained unaffected during the digestion of the oligosaccharide component of ribonuclease B with *A. saitoi* α(1-2)mannosidase.

Table 1. The relative proportions of the glycoforms of ribonuclease B determined by (a) mass spectrometry, (b) Bio-Gel P-4 gel filtration, (c) high performance anion exchange chromatography with radioactive detection, and (d) CE of the glycoprotein. The percentages were obtained by integration of the data in Figs 1–4..

	(*a*) *MS* (%)	(*b*) *P-4* (%)	(*c*) *HPAEC* (%)	(*d*) *CE* (%)
Man_5	47	49	49	48
Man_6	21	19	19	20
Man_7	11	11	10	11
Man_8	16	17	16	17
Man_9	5	4	6	4

The correlation between the two sets of data also confirms that the hydrazinolysis procedure has released the N-linked oligosaccharides of ribonuclease B in molar proportions.

The time course for the digestion of the released sugars with *A. saitoi* α(1-2)mannosidase monitored by HPAEC (Fig. 3) shows the reduction of the Man_9–Man_5 structure to a single Man_5 structure after 34 min. The time course for the digestion of the sugars on the intact glycoprotein under the same conditions and monitored by CE (Fig. 5) shows that after 25 h the glycoform populations carrying Man_9–Man_6 structures were all reduced to a single population carrying Man_5.

Discussion

Ribonuclease B is a glycoprotein (mol wt approx 15,500) containing a single N-glycosylation site at Asn 34. In

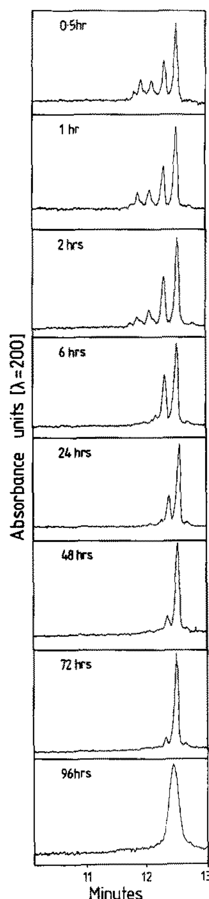

Figure 5. Capillary electrophoresis profiles of ribonuclease B showing the time course for the digestion of the glycoprotein with *A. saitoi* α(1-2)mannosidase. Absorbance at 200 nm is plotted against elution time for time points between 0 and 25 h. This demonstrates that the five glycoforms initially present are reduced by the enzyme to a single structure, the one having the mannose-5 sugar.

common with other glycoproteins it consists of a population of glycosylated variants in which a single amino acid sequence is diversified by the range of oligosaccharides conjugated to it. For a protein containing a single glycosylation site the number and relative proportions of the different glycoforms is equal to the number and relative proportions of the different oligosaccharides associated with the site. This concept allows such glycoform populations to be characterized indirectly in terms of their oligosaccharide structures.

Analysis of the sugars released by hydrazine from RNAse B revealed that this glycoprotein consists of a mixture of five discrete populations each characterized by one of the oligomannose structures from the series Man_9 to Man_5. The relative proportions of these glycoforms correlated with the percentages of the five populations observed when the intact glycoprotein was resolved by capillary electrophoresis, confirming that this technique offers a direct method of analysing glycoforms at the protein level.

The concept of direct and indirect analysis of glycoforms may be usefully applied to the study of other glycoproteins, since it overcomes a common difficulty in the interpretation of CE data where there is rarely enough material for a second dimension analysis. In this respect it is important to establish, as do these data, that the hydrazinolysis procedure releases N-linked oligosaccharides in their correct molar proportions.

In addition to defining the glycoform populations, capillary electrophoresis was used to monitor alterations in these populations directly at the protein level. The oligomannose residues on the intact glycoprotein were digested with *A. saitoi* α(1-2)mannosidase to a single species having a Man_5 structure. Interestingly, the rate of digestion of the free sugars under the same conditions was more than ten times faster than the rate of digestion of intact RNAse B, supporting the concept that the protein may have a role in creating site specific patterns of glycosylation by virtue of its 3D structure interacting with processing enzymes during oligosaccharide biosynthesis.

This novel application of capillary electrophoresis to the analysis of the glycosylated variants of ribonuclease B may represent a useful advance in the technology available for the study of variations in glycoform populations which are known to be specific to the cell type and tissue in which the glycoprotein is produced, and in some cases relate to physiological state and to function.

When glycoproteins are manufactured *in vitro*, the nature of the glycosylation may differ both from the naturally occurring substance and from previous preparations. If a complete analysis of glycosylation is not possible or appropriate such a sensitive 'fingerprinting' approach may indicate whether or not significant glycosylation changes have occurred. Indeed, a recent study has used CE to measure the ratios of different glycoform populations of erythropoietin [13]. Further research will be needed to establish whether this technique can be developed to fingerprint more complicated glycoproteins.

In conclusion, this work has also demonstrated that capillary electrophoresis is a simple and fast way of following an enzymatic digestion of the oligosaccharides associated with a glycoprotein, and this may allow further insight into the role of the protein during glycosylation.

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